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Original Article

AN ISOLATION, IDENTIFICATION AND DIVERSITY OF ENDOPHYTIC FUNGI FROM CATHARANTHUS ROSEUS AND SCREENING FOR THEIR L-ASPARAGINASE ACTIVITY

PUSHPA. H[1], KAVYA. S[2], POOJA. K[2], SNEHA. L[2] & VARSHA O ARER[2]

[1]*Associate Professor and Head, Department of Microbiology, Ramaiah College of Arts, Science and Commerce, Bangalore, India*

[2]*Post Graduate Students, Ramaiah College of Arts, Science and Commerce, Bangalore, India*

ABSTRACT

Catharanthusroseus is one of an important medicinal plant the alkaloids produced by this plant has been used in many medical applications. Many researchers have also isolated the endophytes found in this plant and also proved that some of the endophytes has the ability to produce the metabolites which the host plant produces. Hence in our present research we have made an attempt to isolate, identify and study the diversity of endophytic fungi in C.roseus and we also tried to screen the L-asparaginase producing endophytes in the same. A total of 13 different fungal isolates, 5 different mycelia sterilia and one Actinomycetes were isolated from C.roseus collected from six different locations. Leaf tissue showed greater diversity of endophytes followed by root and stem. This study suggests that endophytes are both host and tissue specific. Preliminary screening of L-asparaginase activity was done for all 58 isolates isolated from C. roseusof which 19 isolates were found to be potential of producing L-asparaginase. All these isolates were subjected for further analysis. The activity of L-asparaginase was analysed by Nesselerization method. Nigrospora sp. followed byLasiodiploidiasp. was found to be potential of producing L-asparaginase. The optimum pH and temperature required for L-asparaginase activity produced by most of the isolates was found to be pH-6 at 30°C. Hence, to the best of our knowledge this is the first report, which revealed the presence of Lasiodiploidia sp. and Madurella sp. as an endophytes in Catharanthusroseus and first report of isolation of L-asparaginase from an endophytic fungi of C.roseus such Nigrospora sp., Madurella sp. and Lasiodiploidia sp. Hence this property of producing such a novel enzyme by these endophytes can be exploited for the production of L-asparaginase which can be further used in pharmaceutical and food industries.

KEYWORDS: Catharanthusroseus, L-asparaginase, Nigrospora sp, Madurella sp, Lasiodiploidia sp & Anticancer Activity

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INTRODUCTION

L-asparaginase is an anticancer drug which has revolutionized the therapy for blood cells related cancers such as acute lymphoblastic leukemia (ALL). Acute lymphoblastic leukemia (ALL) is a malignant transformation of a clone of cells from bone marrow where early lymphoid precursors proliferate and replace the normal cells of the bone marrow. The presence of L-asparaginase depletes the levels of serum L-asparagine it takes asparagine and removes its amine, releasing aspartate and ammonia. Most cells in our body use the enzyme asparagine synthase to make their own asparagine. In normal cells, the asparaginase used for protein synthesis is generated from aspartate

by asparagine synthase. Outside the cell asparagines is converted into aspartate by Asparaginase. L- asparaginase causes selective toxicity for tumor cell because they lack L- asparaginase synthase. L- Asparaginasecatalyses the hydrolysis reaction to convert asparagines into L aspartate and ammonia. L-asparaginase is a relatively wide spread enzyme, found in many microorganisms such as *Aerobacter sp., Bacillus, Pseudomonas fluorescence, Serratia, Xanthomonas, Photobacterium, Proteus, Vibrio, Saccaromyces, Aspergillus, Penicillium, Fusarium*(Patil *et al.,* 2012*;* Curran *et al.,* 1985*;*, Saquis *et al.,* 2004*) Chlamydomonas*sp. *(*Paul, 1982*).* Endophytic organisms have received considerable attention as they protect their host against insect pest, pathogens (Weber, 1995). They are also considered as good source of secondary metabolites like alkaloids, trepenoid, steroids, phenols, peptides, quinines, phenolic acids and flavanoids, these has great therapeutic and medicinal values against many diseases. *Catharanthusroseus*, a popular ornamental plant, belonging to the family Apocyanaceae, has good medicinal properties (Gajalakshmi*et al.,* 2013) this plant is a native of Madagascar, it is also found in India, commonly called as periwinkle. The plant is well known to produce a lot of important compounds especially vinca alkaloids such as vinblastine and vincristine (Manganey*et al.,* 1979). Beside this plant also produces Vincristine, vindoline and catharanthine which are the major monomer alkaloids as well as a biosynthetic precursor for vinblastine (Noble, 1990). The endophytes found associated with this plant for a part of their life cycle without causing any apparent disease symptoms in the host (Petrini, 1991). Some of the endophytic fungi in *Catharanthusroseus* have also been reported by various workers *(*Ayob *et al., 2016); (*Prerna *et al., 2013).* Many for these endophytes have anticancer activity as reported by (Ayob*et al*., 2017). It has also been reported that *Nigrosporasphaerica* an endophytic Fungi in *C.roseus*has a potential of producing vinblastine which is one of an important compound produced by *C.roseus* and has be used in the treatment of cancer. Ashutosh*et al.,* (2013) identified *Fusariumoxysporum* in *C.roseus* could produce vinblastine and vincristine as the host plant produce. In the same way Sreekanth*et al.,* 2017 has reported the presence of *Botryospaeria*sp, *Guignardia* sp. and *Cladosporium* sp. *Alternaria* and *Phoma* sp. present as an endophytes in *C.roseus* and also reported their activity as antimicrobial compound. In the present study, we have made an attempt to study the diversity of endophytic fungi and actinomycetes present in *Catharanthusroseus* collected from various locations. And also to screen L-asparaginase producing endophytes, where L-asparaginase is one of an importance source which has a good anticancer activity.

MATERIALS AND METHODS

Collection of Plant Samples

Healthy, fresh plant of *Catharanthusroseus* was uprooted for soil from four different locations in Bangalore, Pune and Goa in the month of October 2017. Three different samples were collected from each location, brought to the laboratory in sterile bags and used for isolation of endophytic fungi from the different parts of the plants.

Isolation of Endophytic Fungi

For the isolation of fungal endophytes the fresh root, stem, leaves and flowers of *C. roseus* were used as explants. Plant parts were rinsed in running water to remove dust debris. After proper washing, parts of the plants were cut into small bits. Isolation of Endophytic fungi was done according to the method described by Arnold et al., (2000); Sreekanth*et al*., (2017), Robert *et al.,* (1978). Surface sterilization of samples was done by stepwise washing with 70% ethanol for 2 min, 3% sodium hypochlorite solution for 3-2min, followed by rinsing in sterile distilled water and then these segments were placed in pertiplate containing Czapek'sDox agar media supplemented with streptomycin in order to inhibit the growth of bacteria. All the inoculated plates were kept for incubation at 26-28°C for 3-5 days and observed for the growth of fungi on

the plant material.

Identification of Fungal Endophytes

After the period of incubation the plates were observed for the growth of fungus on the explants. The fungus was identified on the basis of morphological (Microscopic and Culture Characteristics) features like colony characterization, growth of fungi, (slow-growing or fast-growing), colour of the colony (Front and reverse), conidial development, size and shape of conidia, the shape of conidial head and attachment of conidia. Barnett 1998 and Gillman 1950 classification concept was used to identify these fungi. The frequency of colonization of each species was calculated by following formula as suggested by Aung*et al.,* (2008). Shannon's diversity index for endophytic fungi in *C. roseus* was calculated as suggested by Margalef*et al.*,(2008). With the help of the values of diversity index, the evenness of the endophytes in C.roseus was also calculates as suggested b yPielou*et al.,* (1996).

Primary Screening of Endophytic Fungi for L- Asparaginase Production

Primary screening of 58 endophytic fungi was done for L-asparaginase production by using a modified protocol as previously described by Patil*et al.,* 2012. In order to check L-asparaginase producers, all the 58 endophytic fungi were screened on Modified Czapek's Dox agar at pH 6. The composition of the Modified Czapek'sDox agar media (L-Asparagine 10g; Glucose 10 g; Sodium nitrate 2g; Ferrous sulphate 0.01g; Magnesium sulphate 05g; Potassium chloride 0.5g; Di-Potassium Phosphate 1g; Agar 18g; Phenol Red 0.009%) Modified Czapek'sDox media was prepared by mixing all the components well except agar and pH was adjusted with 0.1N HCL to 6, then agar was mixed and media was autoclaved at 121°C, 15psi for 15mins. After that media was allowed to cool down, filter sterilized L-asparagine and phenol red was added into it and mixed properly. The plating was done and the plates were kept overnight at 28°C. 7days old culture was inoculated on Modified Czapek'sDox agar plates. The plates were then kept at 28±2°C for 5-7days and were for formation of the pink zone around each colony after the period of incubation.

Secondary Screening of Fungal Isolates by Agar Well Diffusion Assay

The cultures selected on the basis of preliminary testing were further screened on L-asparagine agar plates containing 0.009% phenol red. L-asparagine acts as sole carbon and nitrogen source. These plates are divided into four quadrants well are made using a cork borer were one of the well is kept as control. The culture filtrate was loaded into each well. The colour change to pink was observed and diameter of the zone was measured (Theantana*et al.,* 2007)

Mass Production by Submerged Fermentation Method

The fungal isolates that showed maximum pink zone formation were selected for further evaluation. The fungi isolates were inoculated into Modified Czapek'sDox broth, and incubated for 7days. After the period of incubation broth was filtered through Whatmann filter paper and this filtrate was used as the crude enzyme source for L-asparaginase assay.

Assay of L-asparaginase

In the assay of L-asparaginase the amount of ammonia released as a result of hydrolysis was measured using Nessler's reagent. The level of L-asparaginase enzyme in the culture filtrate was determined using the method of Imada*et al*., (1973). The rate of hydrolysis was determined by measuring the ammonia released using Nessler's reagent. A mixture of 0.5ml of enzyme extract, 0.5ml of 0.04M L-Asparagine, 0.5ml of 0.05M Tris-HCL buffer (pH7.2) and 0.5ml

of distilled water was incubated at 27°C for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA).

The ammonia released into the supernatant was determined spectrophotometrically. To 3.7ml of distilled water, 0.1 of above mixtures and 0.2ml of Nessler's reagent were added and incubated at room temperature for 20min. The absorbance was read at 450nm. Mixture without L-Asparagine was used as blank. One international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate 1µmol of ammonia per minute under the conditions.

Effect of Temperature and pH on L-Asparaginase Activity

Microbial production is affected by parameters like temperature, pH and also affected by carbon and nitrogen sources. The optimization of growth conditions of microorganisms is of prime importance in the production of enzyme which impacts on the economy, yield and productivity as described by Noura*et al.*, 2016.

Isolates which were showing very good activity of asparaginase were selected for further study. Production of L-asparaginase was done by submerged fermentation technique using modified Czapek'sDox Broth. After 7 days the broth culture was filtered using Whatmann filter paper and the culture filtrate was used as a crude extract. The L-asparaginase activity was quantified at different pH such as pH-5, pH-6, pH-7 and pH8. 0.1% L-Asparagine was used as a substrate and incubation temperature ranging from 25°C to 40°C with the incubation period of 30 minutes. The absorbance was read at 450nm and the OD value was plotted on the standard graph of Ammonia to obtain the activity of L-asparaginase.

RESULTS AND DISCUSSIONS

Isolation and Identification of Endophytic Fungi and Actinomycetes from Catharanthusroseus

Catharanthusroseus was collected from different locations in Bangalore, Pune and Goa "Figure 01". Root, stem, leaf and flowers of *C. roseus* were the samples used to isolate fungal endophytes as described by Arnold et al., (2000); Robert *et al*., (1978); Ravi raja*et al*., (2005) and Tiwari, (2012). Isolated endophytic fungi was identified "Figure 02 and 03" on the basis of morphological (microscopic and culture characteristics) features like colony characterization, growth of fungi, (slow-growing or fast-growing), colour of the colony (front and reverse), conidial development, size and shape of conidia, the shape of conidial head and attachment of conidia as described by Barnett and Hunter 1998 and Gilman 1950. A total of 13 different fungal genera and one Actinomycetes (*Acremonium*sp. *Lasiodiploidiatheobromae*, *Alternaria* sp. *Fusarium* sp. *Saccaromyces*sp. *Phoma* sp. *Cladosporium* sp. *Dicoccum* sp. *Penicillium* sp. *Nigospora* sp. *Chrysosporium* sp. *Aspergillus*sp. *Madurella* sp.) and 5 different Mycelia sterilia and 01 Actinomycetes (*Streptomyces* sp.) were isolated from *C.roseus* collected from six different locations.

Figure 1: Habit- Catharanthusroseus

Figure 2: Isolation of Endophytes from C. roseus

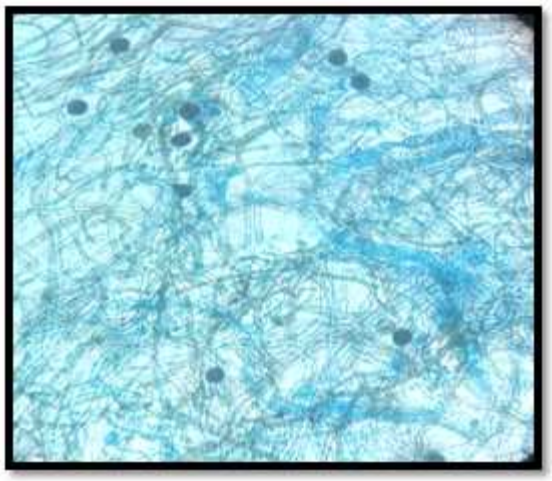

Figure 3: Microscopic View of Nigrospora sp.

A total of 13 different fungal genera interestingly 12 of these belongs to sub divisionDeuteromycotina (*Acremonium*sp. *Lasiodiploidiatheobromae*, *Alternaria* sp. *Fusarium* sp. *Phoma* sp. *Cladosporium* sp. *Dicoccum* sp. *Penicillium* sp. *Nigospora* sp. *Chrysosporium* sp. *Aspergillus*sp. *Madurella* sp.), oneAscomycotina (*Saccaromyces* sp.) 5 different Mycelia sterilia and one Actinomyces (*Streptomyces* sp.) were isolated from *C.roseus* collected from six different location. The presence of these fungal isolates have been well documented by Farah*et al*., 2016 and 2017; Sreekanth*et al.*, 2017. Among these *Lasiodiploidiatheobromae* and *Dicoccum* sp. showed the highest colonizing frequency (15.51% and 13.79%) followed by *Streptomyces* sp. and *Nigrospora* sp. (10.34%), Mycelia sterilia (6.89%), *Cladosporium* sp. (6.89%) *Fusarium* sp., *Aspergillus*sp. (5.17%), and *Alternaria* sp. (3.44%) the lowest was found to be *Penicillium* sp. and *Madurella* sp. (1.72%). *Lasiodiploidia* sp., *Dicoccum*sp., *Nigrospora* sp. and Mycelia sterilia were found to be common in all the sites *Madurella*sp. was found only from *C. roseus* collected form Goa. *Acremonium* sp. and *Alternaria*sp. was found only in *C. roseus* collected from Jalahalli, Bangalore. *Phoma*sp. and *Penicillium*sp. were observed only from Mahalakshmi layout, Bangalore. *Chrysosporium*sp. was isolated from *C. roseus* collected from Pune. Species richness was found to be higher in *C. roseus* collected from Jalahalli, Bangalore and Mahalakshmi layout, Bangalore where the percentage of colonizing unit was found to be 21.8% followed by Indiranagar, Bangalore (18%), MSR Nagar (16.36%), Goa (14.54%) and least was found in Pune (12.72%) as shown in “Figure 04 and 05”. Leaf tissue showed greater diversity of endophytes followed by root and stem. This study suggests that endophytes are both host and tissue specific. It also confirms that despite ecological variation, there were little differences in the species richness of fungal endophytes

recovered from plants in all the sites.

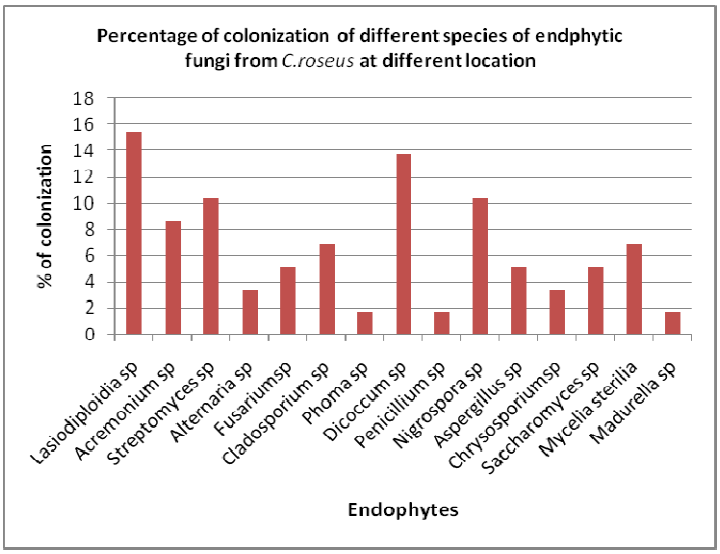

Figure 4: Percentage of Colonization of Endophytic Fungi from Cathranthusroseus at Different Sites

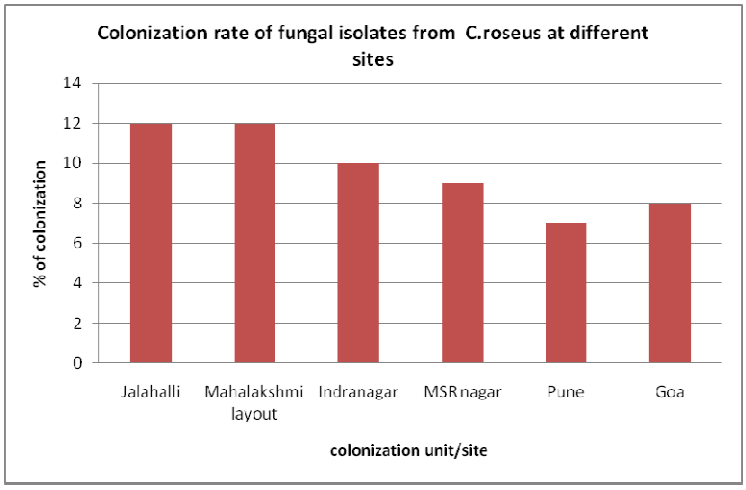

Figure 5: Colonization Rate of Fungal Isolates from Catharanthusroseus at Different Sites

Table 1: Diversity of Endophytes from C.roseus Collected from Different Sites

	BJ	BM	BI	BR	PW	GP
No of species	12	17	10	9	7	7
Total no of individuals	858	294	90	360	252	294
Simpson's diversity index	0.84	0.971	0.82	0.978	0.83	0.023
Shannon diversity index	1.8	1.614	1.3	1.573	1.99	1.925
Evenness	0.0818	1.91	1.3	1.64	2.35	2.24

BJ-Jalahalli, Bangalore; BM-Mahalakshmi layout, Bangalore; BI-Indiranagar, Bangalore; BR-MSR Nagar, Bangalore; PW-Pune; GP-Goa

Primary Screening of Endophytic Fungi Producing L-Asparaginase

58 Isolates of endophytes from *Catharanthusroseus* were subjected to primary screening to evaluate the probability to utilize L-asparagine as sole nitrogen source. The isolates were grown on Modified Czapek's Dox media

where phenol red was used as an indicator and plates were incubated at room temperature for 4-5days. The positive isolates were selected based on the colour change i.e. pink zone around the colony as shown in the **Figure 6** the colour change is due to the accumulation of ammonia which is produced as a result of conversion of L-asparagine to L-aspartic acid.

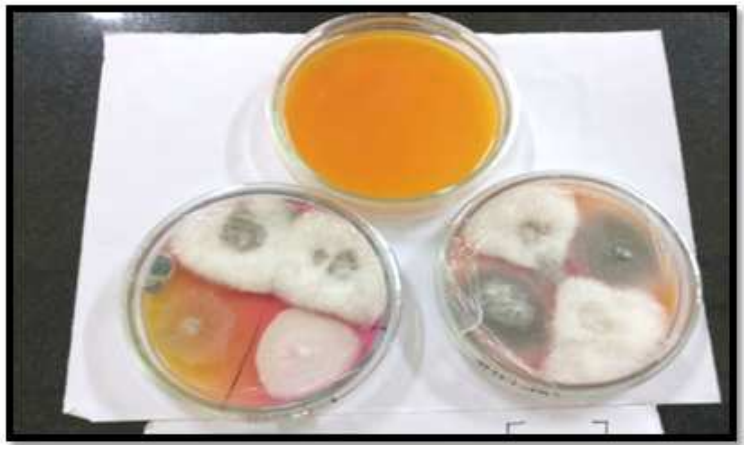

Figure 6: Primary Screening of Endophytic Fungi Producing L-asparaginase

Out of 58 isolates, 32 were found to be positive for extracellular asparaginase production. The 32 positive isolates were *Alternaria*sp. (01), *Acremonium*sp. (02), *Lasiodiploidia*sp. (05), *Streptomyces* sp. (02), *Fusarium*sp. (02), *Saccaromyces*sp. (02), *Cladosporium* sp. (07), *Phoma*sp. (01), *Dicoccum*sp. (05), *Nigrospora* sp. (02), *Madurella*sp. (01), *Chrysosporium*sp.(02), *and Aspergillus* sp. (01). 19 isolates showed positive results for L-asparaginase production within 2 to 3 days of incubation, 16 isolates showed satisfactory L-asparaginase production and the rest were slow producers. The maximum enzyme activity were from *Lasiodiploidia*sp. *Cladosporium*sp. *Dicoccum*sp. and rest were very slow producers being active after seven to ten days as shown in Figure 05.

Secondary Screening of Fungal Isolates by agar Well Diffusion Assay

The positive isolates from primary screening were selected and were grown on Modified Czapek'sDox broth and incubated for 5-6days. The broth was filtered using Whatmann's filter paper No.1 and filtrate was collected. In secondary screening agar well diffusion technique was employed where 10µl of filtrate were dispensed into the wells made on Modified Czapek'sDox plates with one control for eliminating the false results. These plates were incubated at 28°C for 3-4 days. After incubation there was a pink zone formation around the well, the diameter of pink zone was recorded as shown in the **Figure 7.**

Out of 32 positive isolates from primary screening only 19 isolates showed positive in agar well diffusion method. The positive isolates were *Alternaria*sp. (01), *Acremonium*sp. (01), *Lasidiploida*sp. (03), *Streptomycin* sp. (02), *Cladosporium*sp. (03), *Dicoccum*sp. (04), *Nigrospora*sp. (01), *Madurella*sp. (01), *Chrysosporium*sp. (01), and *Aspergillus*sp. (02). Uzma*et al., (*2016) reported that fungal endophytes such as *Fusarium*sp. *Aspergillus* sp. *Lasidiploida*sp. *Nigrospora*sp were isolated from medicinal plants showed extracellular enzyme activity on solid media of which all *Aspergillus*sp was identified as potent isolate for L-asparaginase. Noura*et al*., (2014) reported L-asparaginase of *Streptomyces* sp. the activity was found to be 49.874 IU/ml. L-asparaginase produced by *Lasidiploida*sp. *Cladosporium* sp. *Dicoccum* sp. *Streptomyces* sp. showed better activityand rest were very slow producers being active after seven to ten days. After incubation there was a pink zone formation, the diameter of pink zone was recorded. The maximum zone was observed in *Dicoccum*sp and least in *Chrysosporium*sp.

Figure 7: Secondary Screening of L-asparaginase Produced by Different Isolates

Assay of L-asparaginase

The enzyme activity of L-asparaginase was estimated from culture filtrates by Nesslerization method (Imada *et al.*, 1973). One unit of L-asparaginase was defined as the amount of enzyme that released 1µmol of ammonia, whose abundance was measured at 450nm using Nessler's reagent. L-asparaginase activity ranged from 40 µmole/ml/min – 0.33 µmole/ml/min showed in **Figure 8** of which *Madurella* sp. showed maximum activity followed by *Nigrospora* sp. *Lasiodiploidia*sp. *Cladosporium* sp. and *Streptomyces* sp. All other isolates showed very less activity. Noura*et al.*, (2014); Mostafa*et al.*, (1979a; 1979b) has reported the activity of L-asparaginase from *Streptomyces* sp. Similarly, there are also reports of production of L-asparaginase from *Aspergillus* sp. (Ali, 1994); *Penicillium* sp. (Mohapatra 1997). Nermien*et al.*,(2016) reported that *Alternaria* sp., *Chaetomium* sp., *Cladosporium*sp. found as endophytes in *With aniasomnifera* are also capable of producing L-asparaginase. According to the available reports the production of L-asparaginase by endophytic *Nigrospora* sp. *Lasiodiploidia* sp. has been reported for the first time.

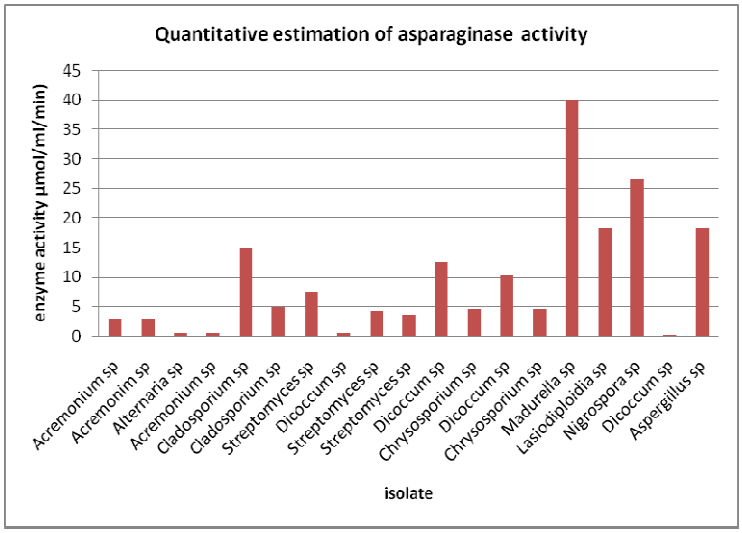

Figure 8: Estimation of L-asparaginase Activity

Effect of pH and Temperature on L-asparaginase Activity

The optimum temperature and pH required for the L-asparaginase activity was also evaluated for the isolates which showed better results. This study revealed that pH 6 at 30°C is the optimum condition required for their maximum activity. Similar reports have been documented by various workers such as Mohan *et al.*(2014). Mrudula*et al.*, (2013)

reported that *Chrysosporium* species showed maximum activity of L-asparaginase at an optimum pH of 4.5 at 50°C. According to Neelima*et al.*, (2014) actinomycetes like *Streptomyces* sp have been reported to produce L-asparaginase within the temperature range of 30-35°C. Hence this work revealed that pH between 5-6 and temperature 30°C is optimum pH and temperature required for L-asparaginase activity however *Cladosporium* sp. and *Streptomyces* sp. shows comparatively better activity even at pH-8 at 25°C.

CONCLUSIONS

The current study has revealed that endophytic fungi can be proved to be a better source of L-asparaginase. Out of 32 isolates of endophytic fungi in *C.roseus*, 19 isolates were found to be a potential producers of L-asparaginase of which *Nigrospora* sp. And *Lasiodiploida* sp. has maximum activity when compared with other isolates. Further enzyme kinetics studies, purification, antitumor activity have to be performed and compared its efficiency with existing commercial L-asparaginase.

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